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Endocytosis in Adult Eel Intestine: Immunological Detection of Phagocytic Cells in the Surface Epithelium

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Abstract. This study was undertaken to isolate groups of eel (*Anguilla japonica*) intestinal cells undergoing active endocytosis *in vivo* and to localize these cells within the intestinal epithelium. A monoclonal antibody (8F1) of the immunoglobulin M class containing kappa light chains was raised against such endocytic cells. Ninety-three percent of the 8F1-positive cells absorbed fluorescent isothiocyanate-conjugated dextran from the intestinal lumen *in vivo* during a 30-min incubation, and they retained the ability to absorb dextran *in vitro*. The 8F1-positive cells constitute about 15% of the whole dissociated, macromolecule-absorbing cells. This suggests that other types of endocytic cells exist in the epithelium. The 8F1-positive cell can internalize fixed *Vibrio anguillarum in vivo*. Immunohistochemical observations demonstrated that 8F1-positive cells are very few in number and are located exclusively in the surface epithelium of the intestine. Thus these cells may well be specified phagocytic cells, different from those playing a basic nutritional role.

Introduction

Intestinal cells of adult teleosts are able to internalize intact protein molecules through pinocytosis (Rombout *et al.*, 1985; McLean and Ash, 1987; Georgopoulou *et al.*, 1988). Furthermore, the intestine ingests the bacterium *Vibrio anguillarum*, the causative agent of a widely distributed hermorrhagic septicaemia of teleost fish (Davina *et al.*, 1982; Rombout *et al.*, 1986; Vigneulle and Laurencin, 1991). A variety of hypotheses meant to explain the physiological significance of these observations have been proposed. Ezeasor and Stokoe (1981) and Georgopoulou *et al.* (1986) have suggested that the non-selective absorption of macromolecules may represent an extension of the normal digestive capacity of the fish intestine. On the other hand, Noaillac-Depeyre and Gas (1976) have proposed that endocytosis may be linked to antigen sampling and the subsequent mounting of an immune response in a similar manner to that described for the microfold or "M" cells of mammals (Owen, 1977; Wolf *et al.*, 1981). But since the cytology of the cells engaged in endocytosis is poorly known, the physiological significance of this phenomenon remains largely a matter for conjecture.

Experiments described in this report were therefore designed to isolate groups of cells undergoing active endocytosis *in vivo* and to determine the location of these cells within the intestinal epithelium. The adult eel *Anguilla japonica* was used as the experimental animal. A monoclonal antibody that reacts specifically with a single group of endocytic cells was obtained. Immunohistochemical analysis with this antibody revealed that a very limited number of the endocytic cells in the surface epithelium of the intestine carries the antigen molecules. The antibody-reactive cell retains the ability to internalize macromolecules *in vitro* after dissociation from the intestine.

Materials and Methods

Materials

Adult specimens of both sexes of the eel *Anguilla japonica*, with body weights of 180–220 g, were obtained

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Abbreviations: ASW, artificial seawater; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FDIC, fluorescein isothiocyanate-conjugated-dextran-incorporating cells; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline without divalent cations; TPBS, PBS containing 0.05% Tween 20.

from a local farm and were maintained without food, in plastic aquaria containing artificial seawater (ASW, 20°C), for more than 1 week before use.

Formalin-fixed cells of the bacterium, *Vibrio anguillarum* strain PT-87050, and rabbit antisera reacting specifically with this bacterial strain were gifts from Dr. T. Nakai, Hiroshima University.

Rabbit immunoglobulin (Ig) G was purified from the serum of a rabbit that had been immunized with a cell membrane fraction of the gastrula of the starfish *Asterina pectinifera* (Ikegami *et al.*, 1991).

Administration of macromolecules and particles into the intestinal lumen and fractionation of intestinal cells

Eel intestines were ligated anteriorly and posteriorly and 0.5 ml of a 1-mg/ml concentration of either fluorescein isothiocyanate (FITC)-conjugated dextran (average molecular mass being 70,000, Sigma), rabbit IgG, or formalin-fixed *Vibrio anguillarum* was injected into the central intestinal lumen. As a control, 0.5 ml of phosphate-buffered saline without divalent cations (PBS, Nissui Seiyaku, Tokyo) was injected similarly. The eels were transferred to an aquarium containing ASW (20°C) and kept for 30 min. After death by decapitation, the eels' intestines were immediately excised, cut along their length, and rinsed in 154 m*M* NaCl. Scraped intestinal mucosa were suspended in 5 ml of PBS containing 5 mg of a mixture of collagenase (0.1 unit/mg) and disperse (0.8 unit/mg) (Boehringer-Mannheim, Mannheim, Germany). After pipetting 20 times with a 2-ml pipet, the suspension was incubated at 25°C for 1 h with constant reciprocal shaking. The suspension was passed through a stainless steel filter (75 μm-mesh), and then centrifuged at 400 × *g* for 10 min. The cell pellet was resuspended in 10 ml of PBS and centrifuged again. This washing process was repeated twice.

The cell pellet was resuspended in PBS at 2.4×10^7/ml. The suspension (0.5 ml) was layered on top of a 12-ml gradient (density: 1.018–1.059 g/ml) of Percoll (Pharmacia), and the cells were separated by centrifugation at 400 × *g* for 1 min. Twelve 1-ml fractions were collected, and the cell number was counted by trypan blue dye exclusion. The number of cells that incorporated FITC-conjugated dextran was examined with a Leitz Orthoplan fluorescence microscope. Cells that incorporated rabbit IgG or formalin-fixed *Vibrio anguillarum* were detected by immunofluorescence microscopy as described below.

Production and characterization of monoclonal antibody

The cell fraction containing the highest percentage of FITC-conjugated-dextran-incorporated cells (FDIC) was washed three times in PBS and resuspended in PBS at 2.8×10^7/ml. A 6-week-old BALB/c female mouse was injected with 1.4×10^7 cells. Three, seven and twelve weeks later, booster injections of 1.5×10^7, 1.8×10^7, and 4.2×10^7 cells, respectively, were administered. Three days after the final booster, the mouse was sacrificed and its spleen was removed. Monoclonal antibody-producing hybridomas were obtained by fusing cells from the spleen with nonproducer myeloma cell line SP-2/O-Ag-14 by the method of Ikegami *et al.* (1991), except that polyethylene glycol 1500 in 75 m*M* Hepes (Boehringer-Mannheim) was used to induce cell fusion, and that Iscove's modified Dulbecco's medium (IMDM, JRH Biosciences) was used instead of Dulbecco's modification of Eagle's medium. The hybridoma culture supernatants were screened by immunofluorescence microscopy as described below. Hybridomas that showed selective reactivity against live FDIC were expanded and subcloned by transferring a single hybridoma to 100 μl of IMDM containing 10% fetal calf serum (FCS, Hezleton Research Products) and 1×10^5 mouse thymocytes.

Types of H and L chains constituting the monoclonal antibody were determined with a mouse monoclonal antibody isotyping kit (Amersham).

Immunofluorescence staining

For screening hybridomas, FDIC were separated as described above, except that the Percoll gradient had a range of buoyant densities, from 1.040 to 1.097 g/ml. To avoid internalization of antibodies, the following procedures were carried out at 4°C. The cell fractions rich in FDIC were washed several times with PBS and then resuspended in 500 μl of hybridoma culture supernatant or preimmune serum and incubated for 20 min. The cells were then washed twice in PBS. Rhodamine-conjugated goat anti-mouse Ig(G + M) (Tago), diluted 1:100 in PBS (0.5 ml), was added to the cell pellet, and the suspension was incubated for 20 min. The cells were spun down and washed three times with PBS. The cells were resuspended in 10 μl of PBS prior to analysis by double-label immunofluorescence microscopy.

FDIC prepared from intestines that had received an injection of PBS, rabbit IgG or fixed *Vibrio anguillarum*, were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature, and were then spun down. The cells were washed twice in PBS and then immersed in methanol (−20°C) for 5 min. The following procedures were carried out at room temperature. The cells were washed three times with PBS containing 0.05% Tween 20 (TPBS) and then immersed in TPBS containing 10 mg/ml bovine serum albumin (BSA) for 30 min, followed by incubation in a hybridoma culture supernatant for 30 min. After three washes in TPBS, the cells were then incubated for 30 min with rhodamine-conjugated goat anti-mouse Ig(G + M) diluted 1:100 in TPBS, washed again three times, and

incubated for 30 min with rabbit anti-*Vibrio anguillarum* antiserum diluted 1:500 in TPBS containing 0.5 mg/ml BSA. This was followed by three more TPBS washes and incubation for 30 min with FITC-conjugated goat anti-rabbit IgG diluted 1:80 in TPBS. The cells were washed three times in TPBS, mounted, and examined by double-label immuno-fluorescence microscopy.

Cells carrying the antigen molecules were detected histologically as follows. Intestines were excised, washed with PBS, and fixed with 4% paraformaldehyde in PBS overnight. The fixed tissue was immersed in 100% ethanol overnight and then embedded in polyester wax (BDH). Sections 7 μm thick were placed onto glass slides and incubated with an appropriate amount of hybridoma culture supernatant for 30 min, after which they were washed in PBS for 15 min. FITC-conjugated rabbit anti-mouse Ig(G + M) (Cappel), diluted 1:80 in TPBS was then added, in the dark, for 30 min, followed by washing with TPBS for 15 min. Slides were mounted in a solution of 50% (volume by volume) in PBS containing 0.1 *M* *n*-propyl gallate to prevent bleaching (Giloh and Sedat, 1982), sealed, and viewed under a fluorescence microscope. All experiments included negative controls in which FITC-conjugated second antibody was used with primary antibody from preimmune serum.

Eel intestines with *Vibrio anguillarum* injected into the intestinal lumen were processed as described above, except that the sections were treated with the following antibodies successively, each time incubating for 30 min, followed by washing with TPBS for 30 min: an undiluted hybridoma culture supernatant; rhodamine-conjugated goat anti-mouse Ig(G + M) diluted 1:100 in TPBS containing 0.5 mg/ml BSA; rabbit anti-*Vibrio anguillarum* antisera diluted 1:500 in TPBS containing 0.5 mg/ml BSA; and FITC-conjugated goat anti-rabbit IgG. The sections were analyzed by double-label immunofluorography.

Extraction of antigen molecules from FDIC and enzyme-linked immunosorbent assay

The cell fractions rich in FDIC (5.0×10^6 cells; density: 1.040–1.047 g/ml) were centrifuged at $400 \times g$ for 5 min. The cell pellet was resuspended in 500 μl of 0.1 *M* potassium phosphate buffer (pH 7.4) containing 1 m*M* phenylmethylsulfonyl fluoride. The cells were disrupted by freeze-thawing six times, and the lysate was centrifuged at $10{,}000 \times g$ for 30 min at 4°C. The supernatant was separated and its protein content was determined by the protein-dye binding assay (Bradford, 1976), or by the method of Lowry *et al.* (1951) with BSA as the standard. Forty microliters of each of a series of serially diluted supernatants, in PBS, were added to a well of a 96-well microtiter plate (Nunc) for enzyme-linked immunosorbent assay (ELISA) and absorbed overnight at 4°C. ELISA was carried out as described in Ikegami *et al.* (1991), except that horseradish peroxidase linked to goat anti-mouse IgM diluted 1:1000 in TPBS was used to locate the first antibody.

The insoluble fraction of the cell lysate was fixed with 1 ml of 2% paraformaldehyde at room temperature for 20 min, followed by centrifugation at $10{,}000 \times g$ for 30 min. The pellet was washed once with distilled water and then treated with 1 ml of methanol (−20°C) for 5 min. Methanol was removed, and the pellet was washed twice with distilled water. The pellet was resuspended in distilled water to give a suspension of 300 μg BSA-equivalent per ml. Forty microliters each of suspensions serially diluted with distilled water were applied to a well which had been previously coated with 0.002% poly-L-lysine and processed in the same way as described for the soluble fraction.

In vitro *incorporation of FITC-conjugated dextran*

The intestinal mucosa was dispersed and fractionated by Percoll gradient centrifugation (density range: 1.040–1.097 g/ml) as described above. The cell fraction rich in the 8F1 positive cells (density range: 1.040–1.047 g/ml) was collected. The cells (2×10^6) were resuspended in 1 ml of PBS, and the suspension was added to 1 ml of 1 mg/ml FITC-conjugated dextran in PBS. The suspension was incubated for 30 min at 20°C in the dark. The cells were washed three times with PBS and resuspended in 2 ml of PBS (4°C). The following procedure was carried out at 4°C. One milliliter of the hybridoma culture supernatant (8F1) was added to the cell pellet, the suspension was allowed to stand for 20 min, and the cells were then washed three times with PBS. One milliliter of rhodamine-conjugated goat anti-mouse Ig(G + M) diluted at 1:100 in PBS was added to the cells. After standing for 20 min, the cells were collected by centrifugation, washed three time with PBS, and analyzed by double-label immunofluorescence microscopy.

Results

Production of monoclonal antibody and the characterization of the antigen molecule

After extensive screening of the hybridoma cell line culture supernatants, a monoclonal antibody that reacted specifically with the surface of one type of living FDIC was obtained and was designated 8F1. As shown in Figure 1, the cell membrane of an FDIC that had incorporated FITC-conjugated dextran *in vivo* reacted to 8F1 at 4°C.

The lysate of FDIC was separated into soluble and insoluble fractions. An ELISA analysis revealed that the soluble fraction contained the antigen molecules. Under the conditions used in this experiment, the maximal re-

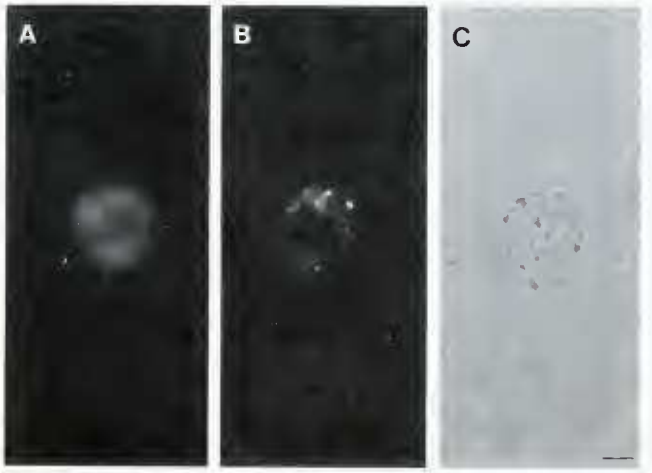

Figure 1. Immunofluorescence micrograph of an intestinal epithelial cell of *Anguilla japonica* incorporating FITC-conjugated dextran *in vivo*. FITC-conjugated dextran (0.5 mg) was injected into the lumen of the intestine. Thirty minutes later, the intestine was excised. The intestinal cells were dissociated, bound to 8F1 antibody at 4°C, and then incubated with rhodamine-conjugated goat anti-mouse Ig(G + M). (A) An endocytic cell, identified by the internal accumulation of FITC-conjugated dextran; (B) the same field showing the 8F1-antigen proteins visualized with the rhodamine-conjugated antibody; (C) the same field observed by brightfield microscopy. Bar, 5 μm.

sponse, which was 1.3 of absorbance at 490 nm, was obtained by applying 3 μg BSA-equivalent of the soluble fraction per well. On the other hand, 6 μg BSA-equivalent of the insoluble fraction per well gave only 15% of the maximum response given by the soluble fraction. When the primary antibody was replaced with preimmune serum, or was omitted, the results were completely negative. We concluded that the antigen molecules were recovered in both soluble and insoluble fractions. When the insoluble fraction was electrophoresed on a reducing sodium dodecyl sulfate-polyacrylamide gel and subsequently analyzed by immunoblot, no 8F1-positive bands were found, whereas proteins of the soluble fraction with apparent molecular masses of 230,000 and 54,000 were stained by 8F1 (data not shown). We must still determine how these soluble 8F1-reactive proteins are related to the antigens located on the cell surface of an FDIC.

The population density of cells harboring 8F1-antigenenic proteins in dissociated intestinal mucosa

FITC-conjugated dextran was introduced into the lumen of the intestine *in vivo*, and the eel was maintained for 30 min before the intestinal tube was excised. The intestinal mucosa were scraped and the cells were dispersed with a mixture of collagenase and disperse. The population density of FDIC was determined by counting fluorescent cells, and 25.5% of the total viable cells were FDIC. Double-label immunofluorescence microscopy, with rhodamine-conjugated goat anti-mouse Ig(G + M) as a second antibody for 8F1, revealed that 15.2% of FDIC (3.9% of the total dissociated cells) and 0.4% of the cells that did not incorporate FITC-dextran (0.3% of the total dissociated cells) were 8F1-positive. When dissociated cells were treated with preimmune mouse serum instead of 8F1, none of the cells observed become fluorescent after the addition of the rhodamine-conjugated secondary antibody (data not shown). These results show that 93% of the 8F1-positive cells were FDIC (Fig. 2).

The dispersed cells were fractionated by Percoll gradient centrifugation. Each cell fraction was fixed, and examined for the presence of 8F1-antigen-containing proteins by immunofluorescence microscopy. There were two groups of FDIC: a comparison with the control tube containing the density marker beads showed that one FDIC fraction had a range of buoyant densities from 1.073 to 1.088 g/ml, and the other FDIC fraction, from 1.040 to 1.047 g/ml. The 8F1-positive cells were found exclusively in the latter fraction (Fig. 3). These results suggested that 8F1 is specific for only one type of endocytic cells in the eel intestine.

The ability of 8F1-positive cells to internalize rabbit IgG and fixed Vibrio anguillarum

The experiments described above were carried out with FITC-conjugated dextran as the ligand for endocytosis. To examine whether the 8F1-positive cell can ingest a proteinaceous ligand as well, 0.5 mg of rabbit IgG was introduced into the intestinal lumen, and the intestine was dissected out 30 min later. The intestinal cells were dispersed, fixed, stained with 8F1, and examined by double immunofluorescence microscopy with FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse Ig(G + M). The majority of 8F1-positive cells incorporated rabbit IgG (Fig. 4). Therefore, the 8F1 positive cells underwent active fluid-phase pinocytosis *in vivo*.

Next we examined whether the 8F1-positive cell could incorporate a particulate ligand. A half milligram of the formalin-fixed bacterium *Vibrio anguillarum* was injected into the lumen of the intestine, and it was processed as described for FITC-conjugated dextran and rabbit IgG. The fixed, dispersed intestinal cells were treated with rabbit anti-*Vibrio anguillarum* antiserum and then analyzed by double immunofluorescence microscopy with FITC-labeled goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse Ig(G + M). Only the 8F1-positive cells incorporated fixed *Vibrio anguillarum* (Fig. 5). When 0.5 ml of PBS instead of a 0.5-ml suspension of fixed *Vibrio anguillarum* in PBS was introduced into the intestinal lumen and the intestinal cells were processed similarly,

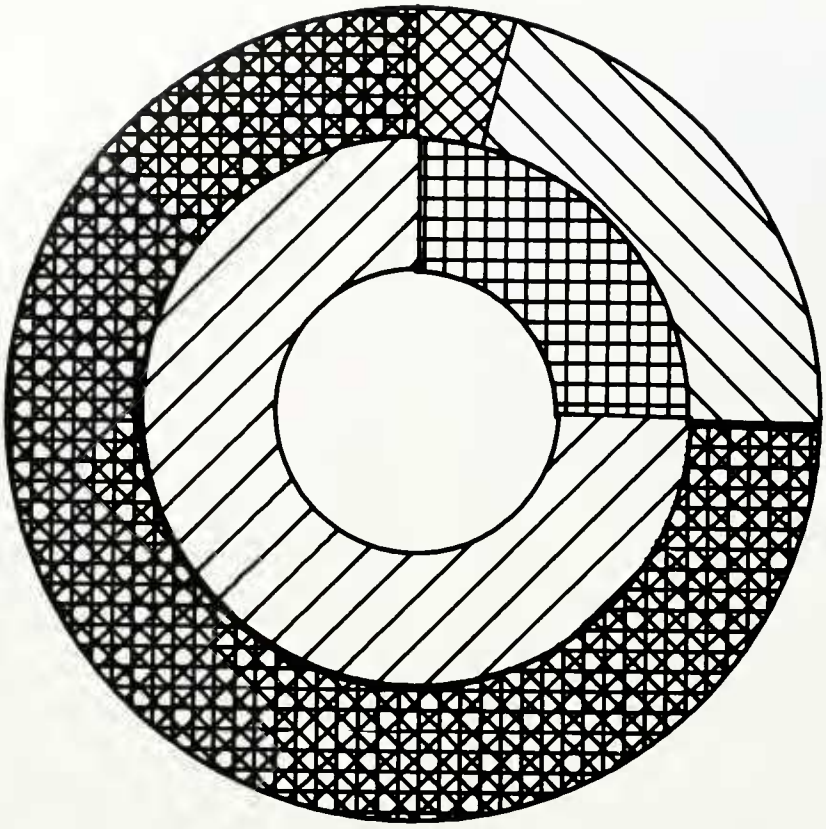

Figure 2. Distribution of 8F1-positive and 8F1-negative FITC-conjugated-dextran-incorporating cells (FDIC) dissociated from the intestine of *Anguilla japonica*. (▦), Percentage of FDIC; (▩), percentage of 8F1-positive FDIC; (▧), percentage of 8F1-negative FDIC; (▨), percentage of cells that did not incorporate FITC-conjugated dextran; (■), percentage of 8F1-positive cells that did not incorporate FITC-conjugated dextran; (■), percentage of 8F1-negative cells that did not incorporate FITC-conjugated dextran.

no specific fluorescence could be detected in the cells after staining.

Localization of 8F1-positive cells in the intestinal epithelium

To localize the 8F1-positive cells in the intestine, the tissue was sectioned and stained with 8F1. Few 8F1-positive cells were present in any villus, and the cells were localized exclusively in the intestinal epithelium (Fig. 6). When a section was stained with the preimmune sera, these cells did not become fluorescent upon the addition of FITC-labeled second antibody (data not shown). When fixed *Vibrio anguillarum* was put in the intestinal lumen, only 8F1-positive cells took up the bacterium; no other cells in the epithelium absorbed this particulate probe under the conditions specified (Fig. 7).

In vitro *uptake of FITC-conjugated dextran by 8F1-positive cells*

To examine whether the 8F1-positive cell could ingest FITC-conjugated dextran *in vitro*, FDIC were prepared, incubated in PBS containing the fluorescent probe for 30 min at 20°C, and then washed extensively. Double immunofluorescence microscopy showed that the 8F1-positive cells took up FITC-conjugated dextran (Fig. 8). When incubation was carried out at 4°C, there was no specific intracellular localization of fluorescent material. These results show that the process of endocytosis is temperature-dependent, and that 8F1 applied to a cell suspension at 4°C complexes with the antigen proteins on the cell surface and remains there without being endocytosed, as is shown in Figure 1.

Discussion

Many reports indicate that enterocytes of teleost fish ingest proteins such as horseradish peroxidase, ferritin, Igs and peptide hormones (Noaillac-Depeyre and Gas, 1976; Rombout *et al.*, 1985; Georgopoulou and Vernier, 1986; Georgopoulou *et al.*, 1986, 1988; McLean and Ash, 1987; Suzuki *et al.*, 1988; Bail *et al.*, 1989; McLean *et al.*, 1990; Moriyama *et al.*, 1990). The experimental fish used in these studies belong to Clupeiformes or to Cyprinbi-

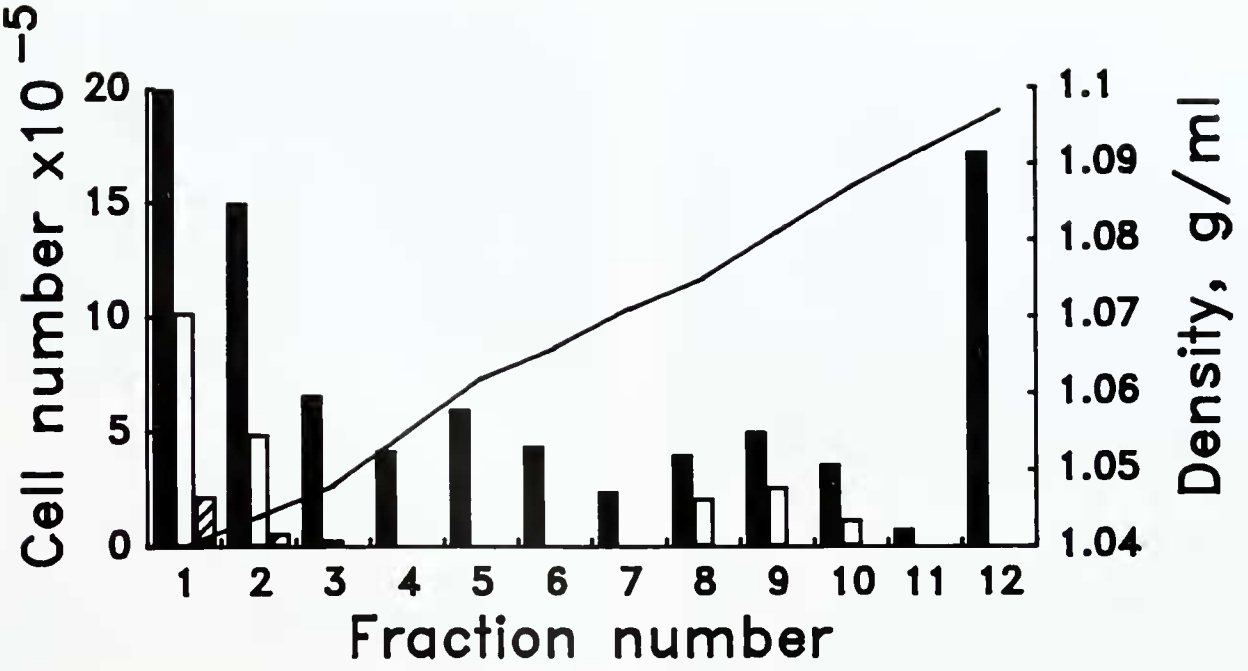

Figure 3. Percoll density-gradient centrifugation of intestinal epithelial cells of *Anguilla japonica*. Procedures for separation are described in Materials and Methods. (■), Number of total cells; (□), number of cells that incorporated FITC-conjugated dextran; (▨), number of 8F1-positive cells; (—), density of Percoll.

formes. Vigneulle and Laurencin (1991) showed that formalin-fixed *Vibrio anguillarum* was ingested by enterocytes of rainbow trout *Oncorhynchys mykiss*, sea bass *Dicentrarchus labrax*, and turbot *Scophthalmus maximus* after oral administration or anal intubation. To our knowledge, the present paper is the first to show that active endocytic cells occur in the intestine of the adult eel. The aim of this study was to prepare monoclonal antibodies to probe for cells undergoing active endocytosis in the eel intestine. We succeeded in obtaining a monoclonal antibody, 8F1, which specifically recognized one type of endocytic cell. We made a survey of cells harboring 8F1-reactive proteins in the histological sections that had been prepared from the intestine of various species belonging to Cluperiformes, Myctophiformes, Cypriniformes, Anguilliformes, Cyprinodontiformes, and Perciformes. Several cells located in the intestinal epithelium of the adult conger eel *Conger myriaster* recognized 8F1, whereas no 8F1-positive cells were found in the intestine of species belonging to other orders than Anguilliformes (manuscript in prep.).

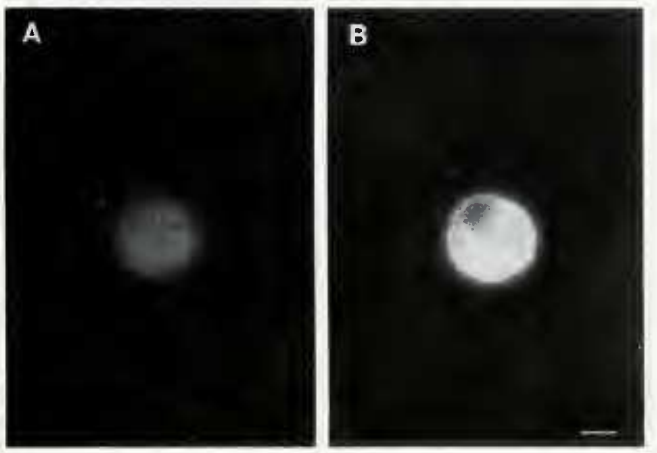

Figure 4. Double-label, immunofluorescence micrograph of an intestinal epithelial cell of *Anguilla japonica* incorporating rabbit IgG *in vivo*. Rabbit IgG (0.5 mg) was injected into the lumen of the intestine. Thirty minutes later, intestinal cells were separated, fixed, and treated with methanol. After being labeled with 8F1, the cells were double-labeled with rhodamine-conjugated goat anti-mouse Ig(G + M) and FITC-conjugated goat anti-rabbit IgG. (A) A cell containing rabbit IgG visualized with the FITC-conjugated antibody; (B) the same cell harboring the 8F1-antigen proteins, identified by the labeling with the rhodamine-conjugated antibody. Bar, 5 μm.

Because the number of 8F1-positive cells in a villus of the eel intestine is very small, such cells are unlikely to play a basic nutritional role. The 8F1-positive cells may be migrating neutrophils or macrophages infiltrated across the blood vessel. But the 8F1-positive cells were found only in the villus epithelium and were absent in the lamina propria, suggesting that they are not such types of leucocytes.

In the intestinal epithelium of mammals, M cells that overlie Peyer's patches have been shown to transport

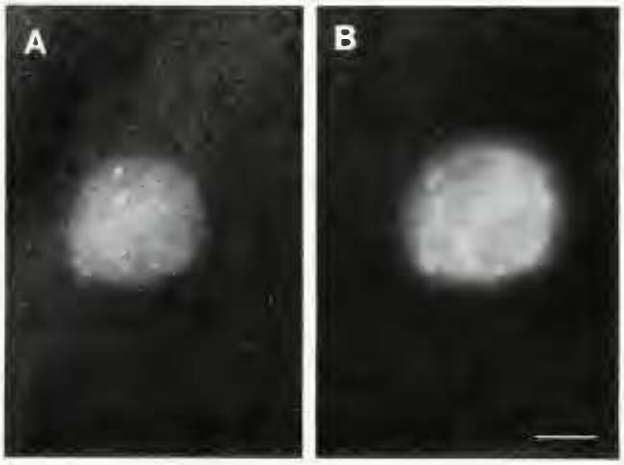

Figure 5. Double-label, immunofluorescence micrograph of an intestinal epithelial cell of *Anguilla japonica* incorporating fixed *Vibrio anguillarum in vivo*. Fixed *Vibrio anguillarum* (0.5 mg) were injected into the lumen of the intestine. Thirty minutes later, intestinal cells were separated, fixed, and treated with methanol. After binding with 8F1 antibody, the cells were labeled with rhodamine-conjugated goat anti-mouse Ig(G + M) and rabbit anti-*Vibrio angullarum* antisera and finally with FITC-conjugated goat anti-rabbit IgG. (A) A *Vibrio anguillarum*-incorporated cell visualized with anti-*Vibrio anguillarum* antiserum and the FITC-conjugated secondary antibody; (B) the same cell harboring the 8F1-antigen proteins, identified by their labeling with the rhodamine-conjugated antibody. Bar, 5 μm.

macromolecules, such as horseradish peroxidase and ferritin (Bockman and Cooper, 1973; Owen, 1977). Furthermore, viruses (such as reovirus) were phagocytosed into the cells (Wolf *et al.*, 1981; Morrison *et al.*, 1991). Such an endocytic process and the subsequent release of the ingested material into the intercellular space is considered to be the origin of a local IgA immune system (Georgopoulou *et al.*, 1988). It remains to be determined whether the 8F1-positive cell can transfer absorbed material towards the intercellular space and into the circu-

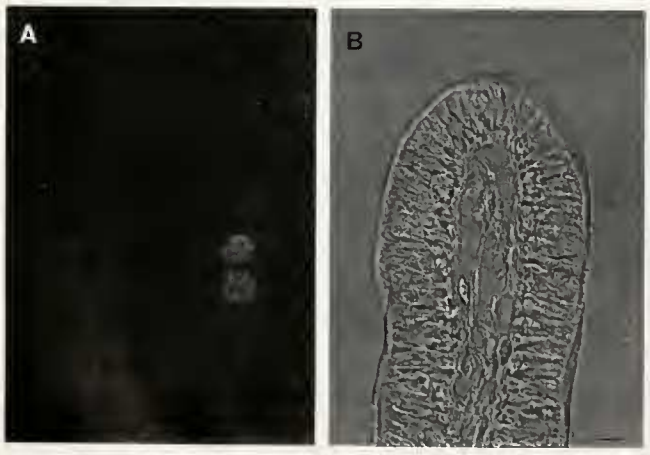

Figure 6. Immunofluorescence micrograph of a section of an intestinal segment of *Anguilla japonica* which was stained with 8F1 and FITC-conjugated rabbit anti-mouse IgG. (A), A section observed by fluorescence microscopy; (B) the same field observed under brightfield microscopy. Bar, 10 μm.

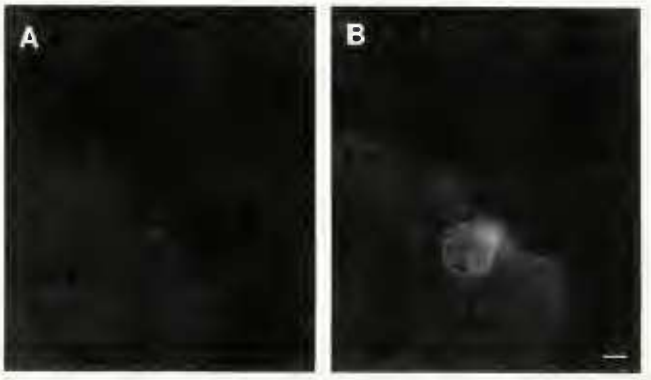

Figure 7. Double-label, immunofluorescence micrograph of a section of an intestinal segment of *Anguilla japonica* incorporating fixed *Vibrio anguillarum in vivo*. Fixed *Vibrio anguillarum* (0.5 mg) were injected into the lumen of the intestine. Thirty minutes later, an intestinal segment was excised and embedded in polyester-wax. Sections (7 μm) were double-labeled with 8F1 antibody and rabbit anti-*Vibrio anguillarum* antibody, with rhodamine-conjugated goat anti-mouse Ig(G + M) and goat FITC-conjugated goat anti-rabbit IgG as second antibodies. (A) A section stained with anti-*Vibrio anguillarum* antibody and the FITC-conjugated secondary antibody; (B) the same field stained with 8F1 and the rhodamine-conjugated second antibody. Bar, 5 μm.

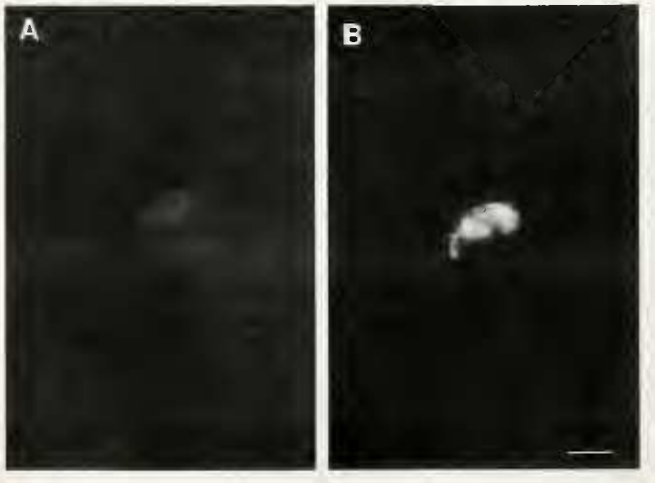

Figure 8. Immunofluorescence micrograph of an intestinal cell of *Anguilla japonica* incorporating FITC-conjugated dextran *in vitro*. Intestinal epithelial cells were dissociated and separated by Percoll density gradient centrifugation. The cell fractions with the range of buoyant densities, 1.040–1.047 g/ml, were collected and incubated with FITC-conjugated dextran for 30 min. They were then immunostained with 8F1 and rhodamine-conjugated goat anti-mouse Ig(G + M). (A) A cell incorporating FITC-conjugated dextran; (B) the same cell harboring the 8F1-antigen proteins, labeled with the rhodamine-conjugated second antibody. Bar, 5 μm.

latory system, thereby triggering local and systemic immune responses. Both histochemical and ultrastructural studies of the 8F1-positive cell are obviously required before the functional resemblance of the cell to M cells of mammals can be discussed.

Acknowledgments

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